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Inactivation, Sequence, and *lacZ* Fusion Analysis of a Regulatory Locus Required for Repression of Nitrogen Fixation Genes in *Rhodobacter capsulatus*

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Transcription of the genes that code for proteins involved in nitrogen fixation in free-living diazotrophs is typically repressed by high internal oxygen concentrations or exogenous fixed nitrogen. The DNA sequence of a regulatory locus required for repression of *Rhodobacter capsulatus* nitrogen fixation genes was determined. It was shown that this locus, defined by Tn*5* insertions and by ethyl methanesulfonate-derived mutations, is homologous to the *glnB* gene of other organisms. The *R. capsulatus glnB* gene was upstream of *glnA*, the gene for glutamine synthetase, in a *glnBA* operon. β-Galactosidase expression from an *R. capsulatus glnBA-lacZ* translational fusion was increased twofold in cells induced by nitrogen limitation relative to that in cells under nitrogen-sufficient conditions. *R. capsulatus nifR1*, a gene that was previously shown to be homologous to *ntrC* and that is required for transcription of nitrogen fixation genes, was responsible for approximately 50% of the transcriptional activation of this *glnBA* fusion in cells induced under nitrogen-limiting conditions. *R. capsulatus* GLNB, NIFR1, and NIFR2 (a protein homologous to NTRB) were proposed to transduce the nitrogen status in the cell into repression or activation of other *R. capsulatus nif* genes. Repression of *nif* genes in response to oxygen was still present in *R. capsulatus glnB* mutants and must have occurred at a different level of control in the regulatory circuit.

Most free-living, unicellular diazotrophs fix nitrogen only in the absence of both oxygen and fixed nitrogen. The enzyme nitrogenase, which reduces atmospheric nitrogen to ammonia, is inactivated by oxygen. In addition, exogenous ammonia or other reduced nitrogen sources make this energy-intense reduction unnecessary. Therefore, bacteria have developed regulatory mechanisms to repress the synthesis of their nitrogen fixation (i.e., *nif*) proteins when either of these two effectors is present.

The paradigm for studies on *nif* gene organization and regulation is *Klebsiella pneumoniae*, a close relative of *Escherichia coli* and *Salmonella typhimurium*. In this diazotroph, two levels of control are present (for a review, see reference 23). The first level of control involves proteins that sense and respond to the intracellular concentration of fixed nitrogen and, presumably, is similar for *K. pneumoniae*, *Escherichia coli*, and *S. typhimurium* (43, 44, 52). This regulation requires the genes *glnD*, *glnB*, *ntrB*, *ntrC*, and *ntrA*. GLND is a uridylyltransferase (4) which uridylylates another protein, GLNB, when the ratio of glutamine to α-ketoglutarate in the cell is low (i.e., low levels of fixed nitrogen). At a high ratio, this same enzyme removes the UMP moiety from GLNB (for a review, see reference 60). When GLNB is not uridylylated, it interacts with another protein, NTRB (35). This interaction results in the removal of an aspartyl-bound phosphate group from NTRC (55, 67). Dephosphorylated NTRC is unable to activate transcription at *ntr* (i.e., nitrogen-regulated) promoters. When GLNB is uridylylated or the *glnB* gene is inactivated, NTRB acts as a kinase and phosphorylates NTRC. The phosphorylated form of NTRC is a transcriptional activator of *ntr*-regulated genes. This activation also requires RNA polymerase containing a specific sigma factor encoded by *ntrA* (27, 30).

One of the *ntr*-regulated promoters in *K. pneumoniae* is the *nifLA* promoter (23, 61). Thus, transcription of *nifLA* begins the second level of control. The *nifLA* operon is 1 of 7 operons comprising at least 17 *nif* genes that are clustered within 24 kilobases (kb) on the *K. pneumoniae* chromosome (1). One of these operons includes the highly conserved *nifHDK* genes that encode the nitrogenase MoFe protein (NIFD and NIFK) and the nitrogenase reductase Fe protein (NIFH). Expression of *nifL* and *nifA-lacZ* fusions are repressed between 80 and 95% when *K. pneumoniae* is grown under highly aerated conditions (37, 45). Transcription of nitrogen fixation genes (41), and specifically *nifLA* (15, 17), has been shown to require optimal DNA supercoiling both in vivo (15, 41) and in vitro (17). These data, together with genetic data (68) and the observation that marker plasmids are more negatively supercoiled in anaerobically than in aerobically grown *Escherichia coli* (18), have led to the suggestion that the *nifLA* promoter is the first point of anaerobic control that is modulated by the level of DNA supercoiling (15, 17, 41). Another point of anaerobic control occurs at the level of the NIFA protein. When oxygen, or apparently fixed nitrogen, is present in *K. pneumoniae*, NIFL inactivates NIFA by an unknown mechanism (8, 25). NIFA is required for the transcriptional activation of all other *nif* operons (51), and like NTRC, NIFA possesses a conserved nucleotide-binding domain (7, 48). Recently, this central domain was shown to be essential for the transcription activation function of NIFA from *Rhizobium meliloti* (29). NIFA binds to a DNA sequence (consensus $TGTN_{10}ACA$) upstream of *nif* operons (49) and, by a mechanism presumably similar to that used by NTRC (29, 55), activates the NTRA-RNA polymerase holoenzyme.

Studies on the regulation of nitrogen fixation genes in the

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photosynthetic bacterium *Rhodobacter capsulatus* indicate that at least four genes are required for activation of *nif* genes. *nifR2*, which is homologous to *ntrB* (2, 34, 40); *nifR1*, which is homologous to *ntrC* (40); and *nifR4*, which is homologous to *ntrA* (2, 34, 40), are all required to activate a *Rhodobacter capsulatus nifH-lacZ* fusion in vivo (40). Another gene, *nifA*, exists in two copies, and a *Rhodobacter capsulatus* strain with insertion mutations in both copies does not synthesize *nif* polypeptides (36, 46). The *Rhodobacter capsulatus nifA* gene has been shown to be homologous to the *K. pneumoniae nifA* gene and to the *nifA* genes from other diazotrophs (46). Expression of *nif* genes in *Rhodobacter capsulatus* also requires optimal DNA supercoiling (41). *Rhodobacter capsulatus* mutants that do not repress *nif* gene expression when fixed nitrogen is present (i.e., $Nif^{c:NH_4}$) but that still repress expression under aerobic conditions have been isolated (41, 42). In a previous study, a *Rhodobacter capsulatus* DNA fragment that returned some of these $Nif^{c:NH_4}$ mutants (e.g., 51nif^c) to wild-type control was isolated and mapped (42). In the study described here, we show that this repressor gene, called *nifR5*, is functionally and physically homologous to the *Escherichia coli glnB* gene described above. Hence, we redesignate *nifR5* as *glnB*. It is upstream of *glnA*, which is the gene encoding glutamine synthetase (GS), in a *glnBA* operon. Its role in sensing fixed nitrogen with respect to the *Rhodobacter capsulatus nif* regulatory circuit is discussed.

MATERIALS AND METHODS

Media and growth conditions. *Rhodobacter capsulatus* basal medium (RCV) and drug concentrations have been described previously (2). When glutamine was necessary, it was added to RCV at a final concentration of 10 mM. Cells for inductions or chromosomal DNA preparations were typically inoculated into 5 ml of medium and grown at 34°C in illuminated, unshaken 20-ml test tubes. Inductions under anaerobic conditions were carried out by adding cells to 12.5-ml stoppered screw-cap tubes and filling them to the top with medium. Inductions under aerobic conditions were carried out by adding 1 ml of culture to 12.5 ml of medium in 250-ml flasks and shaking the flasks at 300 rpm for approximately 15 h. When cells were induced in different media, they were first washed in RCV without ammonia and then suspended in induction medium.

Bacterial strains and plasmids. The *Rhodobacter capsulatus* strains used in this study are described in Table 1. SB1003 is a spontaneous rifampin-resistant strain (69), and J61 and LJ1 are Nif^- mutants that have been described previously (64, 66) and have been shown to be mutants in the regulatory genes *nifR1* and *nifR4*, respectively (2, 40). Strains 51nif^c, 53nif^c, and 54nif^c have been described previously (41, 42). KR548, a strain possessing a Tn*5* insert in the chromosomal *Rhodobacter capsulatus glnB* (*nifR5*) gene, was isolated in the following manner. Plasmid-borne *glnB*::Tn*5* (in pRCN548), which has been described previously (42), was recombined into the chromosomal *glnB* gene by using the incompatible gentamicin-resistant (Gen^r) plasmid pPH1J1. All media used in this isolation contained 10 mM glutamine, and exconjugate recombinants were selected aerobically for Gen^r and kanamycin resistance (Kan^r) on RCV plates containing glutamine.

pRK1218 and pRK618 were constructed by cloning, in both orientations, the 2.2-kb *Eco*RI fragment that contained *Rhodobacter capsulatus glnB* into pUC118 (63). A *Rhodobacter capsulatus glnBA-lacZ* fusion plasmid, pRKR5GS, was constructed in the following manner. pRK1218 was digested with *Pst*I to yield a 4.8-kb fragment that cut within the *glnA* gene (see Fig. 1). The 6.2-kb *Escherichia coli lacZYA Pst*I fragment from pSKS105 (10) was ligated to this fragment, and pRKR5A was determined to possess the correctly oriented *glnBA-lacZ* fusion. The *lacZYA Pst*I fragment from pSKS105, when ligated into the *Pst*I site in *glnA*, was predicted from the DNA sequence data to create an in-frame *glnA-lacZ* translational fusion. An *Eco*RI-*Sph*I fragment in pRKR5A that possessed only the 1.8-kb *glnBA* region fused to the 6.2-kb *lacZYA* region was cloned into pUCA6 by digesting pUCA6 with *Hin*dIII and *Sph*I to yield an 8.25-kb vector fragment; it included the 54-base-pair (bp) *Hin*dIII-*Eco*RI pUC119 polylinker frag-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Rhodobacter capsulatus strains		
SB1003	Wild type	69
51nif^c	*nifH-lacZ glnB*; *nif*(Con) with respect to ammonia	41
53nif^c	*nifH-lacZ glnB*; *nif*(Con) with respect to ammonia	41
54nif^c	*nifH-lacZ glnB*; *nif*(Con) with respect to ammonia	41
J61	*nifR1* mutant	64
LJ1	*nifR4* mutant	66
KR548	*glnB*::Tn*5* chromosomal insert	This study
Plasmids		
pLAFR1	Tet^r cosmid vector (mobilizable by pRK2013)	22
pRCN300	Contains *nifR5* (i.e., *glnB*) gene in pLAFR1	42
pUCA6	10-kb Tet^r cosmid vector (mobilizable by pRK2013); RK2 origin of replication	W. Buikema and J. Meeks
pRK2013	Kan^r	16
pRCN548	pRCN300 with Tn*5* in *nifR5* (i.e., *glnB*) gene	This study
pRK1218	Amp^r; 2.2-kb *Eco*RI *Rhodobacter capsulatus glnB* fragment in pUC118	This study
pRK618	Amp^r; 2.2-kb *Eco*RI *glnB* fragment in opposite orientation in pUC118 than it is pRK1218	This study
pRKR5A	*Rhodobacter capsulatus glnBA-lacZ* fusion in pUC118	This study
pRKR5GS	*Rhodobacter capsulatus glnBA-lacZ* fusion in pUCA6	This study
pPH1J1	Gen^r (incompatible with pLAFR1-derived plasmids)	26
pSKS105	Amp^r; contains *Escherichia coli lacZYA* operon	10
pUC118	Amp^r; possesses M13 intergenic region	63
pUC119	Amp^r; possesses M13 intergenic region	63

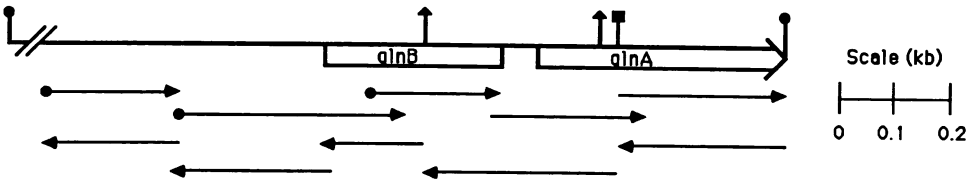

FIG. 1. Restriction map and sequencing strategy of the 2.2-kb *Eco*RI fragment containing *glnB* (i.e, *nifR5*) and part of *glnA*. Clones were constructed as described in the text. Horizontal arrows indicate the direction and extent of each clone that was sequenced. Horizontal arrows with a closed circle mark positions where a synthetic oligonucleotide was used for DNA sequencing. Restriction enzyme sites are indicated by vertical lines with the following symbols: ●, *Eco*RI; ▲, *Sal*I; ■, *Pst*I.

ment in the ligation. The resulting tetracycline-resistant (Tet^r) broad-host-range plasmid, pRKR5GS, showed the restriction digest patterns that were expected for the *glnBA-lacZ* fusion in pUCA6. The construction of pUCA6 (W. Buikema, University of Chicago, and J. Meeks, University of California, Davis) will be described elsewhere.

DNA sequencing. DNA sequencing was carried out by the dideoxy method of Sanger et al. (58), and [^{35}S]ATP sequencing was carried out with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). The sequencing strategy is shown in Fig. 1. All sequencing was carried out by using single-stranded templates prepared from plasmids with inserts in pUC118 and pUC119 (63). These plasmids were constructed by linearizing pRK1218 and pRK618 with *Bam*HI followed by partial digestion with *Sau*3A. A set of plasmids with ordered deletions was constructed by religating size-selected linear fragments. The approximate size of each deletion was determined by digestion with appropriate restriction enzymes. Some templates were prepared from plasmids with specific restriction fragments cloned into pUC118 or pUC119. Synthetic oligonucleotides were used as primers in some regions (Fig. 1).

Southern hybridization. Chromosomal DNA was prepared from cells by a procedure described previously (38). Southern blot hybridizations were carried out at 65°C as described previously (40), except that GeneScreen Plus (Du Pont Co., Wilmington, Del.) was used instead of nitrocellulose and DNA was transferred for 2 h by using 0.4 M NaOH instead of SSPE. Probes were made by separating the DNA fragments that were to be labeled in a 1% low-melting-temperature agarose gel. The fragment was excised, boiled for 5 min in a 0.5-ml microfuge tube, and cooled to 37°C for 5 min. A complete random primer oligo mix (19) of equal volume, also at 37°C, was added. Finally, [^{32}P]dCTP and the DNA polymerase Klenow fragment were added in concentrations previously recommended for random primer labeling (19). The mix was allowed to gel and incubate at room temperature overnight. This was boiled for 5 min and used directly in the Southern blot.

Other methods. Conjugations with triparental matings were carried out as described previously (2). Western immunoblots with *Rhodospirillum rubrum* nitrogenase reductase Fe protein antiserum (kindly provided by Paul Ludden, University of Wisconsin, Madison) and *Anabaena* sp. strain GS antiserum (kindly provided by R. Haselkorn, University of Chicago) were performed as described previously (39). β-Galactosidase activities and protein measurements by the Lowry method were determined as described previously (40).

RESULTS

***Rhodobacter capsulatus nifR5* is homologous to *Escherichia coli glnB* and proximal to *glnA*.** The approximate location of *nifR5* was previously determined by Tn*5* inactivation of its activity (42). *Rhodobacter capsulatus* 51nif^c, 53nif^c, and 54nif^c possessed mutations in this *nifR5* locus (42). The end of the 2.2-kb *Eco*RI DNA fragment that included *nifR5* was sequenced by the strategy shown in Fig. 1. The sequence of 1,360 bp was determined (Fig. 2). In this sequence, three open reading frames (ORFs) that showed typical *Rhodobacter capsulatus* codon frequencies (i.e., rare third-position A or T residues) were found. The region previously defined by Tn*5* inactivation of *nifR5* (42) started at 548 bp and ended at 886 bp. The sequence of a second ORF began 83 bp distal to *nifR5* and was translated in the same direction as *nifR5*. Only a portion of this gene was sequenced (up to the *Eco*RI site). Both the *nifR5* ORF and the distal ORF showed putative ribosome-binding sites upstream of potential ATG start codons that were similar to Shine-Dalgarno sequences of other *Rhodobacter capsulatus* genes (14). A third potential ORF, proximal to *nifR5*, would be translated in the opposite direction to *nifR5*. Only a portion of it was sequenced (from 356 bp to the beginning of the sequence; Fig. 2). All ORFs were compared with the GenBank and NBRF data bases by using TFASTA and FASTA software, respectively (53). The ORF (i.e., N-terminal portion) proximal to *nifR5* showed no significant homology to other proteins in the data bases. The ORF distal to *nifR5* (i.e., N-terminal portion) showed significant homology to the GS gene, *glnA*, from *Escherichia coli* (11, 47), *S. typhimurium* (31), *Azospirillum brasilense* (5), *Rhizobium leguminosarum* (12), and *Anabaena* spp. (62) (data not shown). The homology of this portion of the *Rhodobacter capsulatus* ORF ranged from 45% identity for the *Anabaena* GS to 53% identity for the *S. typhimurium* GS. *nifR5* showed significant homology to DNA sequences from *Rhizobium leguminosarum* (12), *Bradyrhizobium japonicum* (9), *Erwinia chrysanthemi* (54), and the *Escherichia coli* (60) *glnB* genes. Comparisons of *nifR5* with these and other published *glnB* sequences are shown in Fig. 3. The *Rhizobium leguminosarum glnB* gene was published as an ORF proximal to a *glnA* gene (12). The *B. japonicum* (9) and *Azospirillum brasilense* (5) sequences were only partial sequences from DNA upstream of *glnA*, and the *Erwinia chrysanthemi* sequence was only a C-terminal partial sequence from a DNA sequence distal to a gene that codes for pectin methyl esterase (54). The complete sequence of the *K. pneumoniae glnB* gene has been described recently (28). All four complete GLNB amino acid sequences were conserved at tyrosine-51, the proposed site of uridylylation (60). Based on these results, we redesignated the *Rhodobacter capsulatus nifR5* gene as *glnB*.

Inactivation of the chromosomal *Rhodobacter capsulatus glnB* gene. Previous attempts to inactivate *Rhodobacter capsulatus glnB* by recombination of Tn*5* insertions into the chromosomal locus were unsuccessful (42). In those experiments Tn*5* inserts in pRCN300 that were shown to inacti-

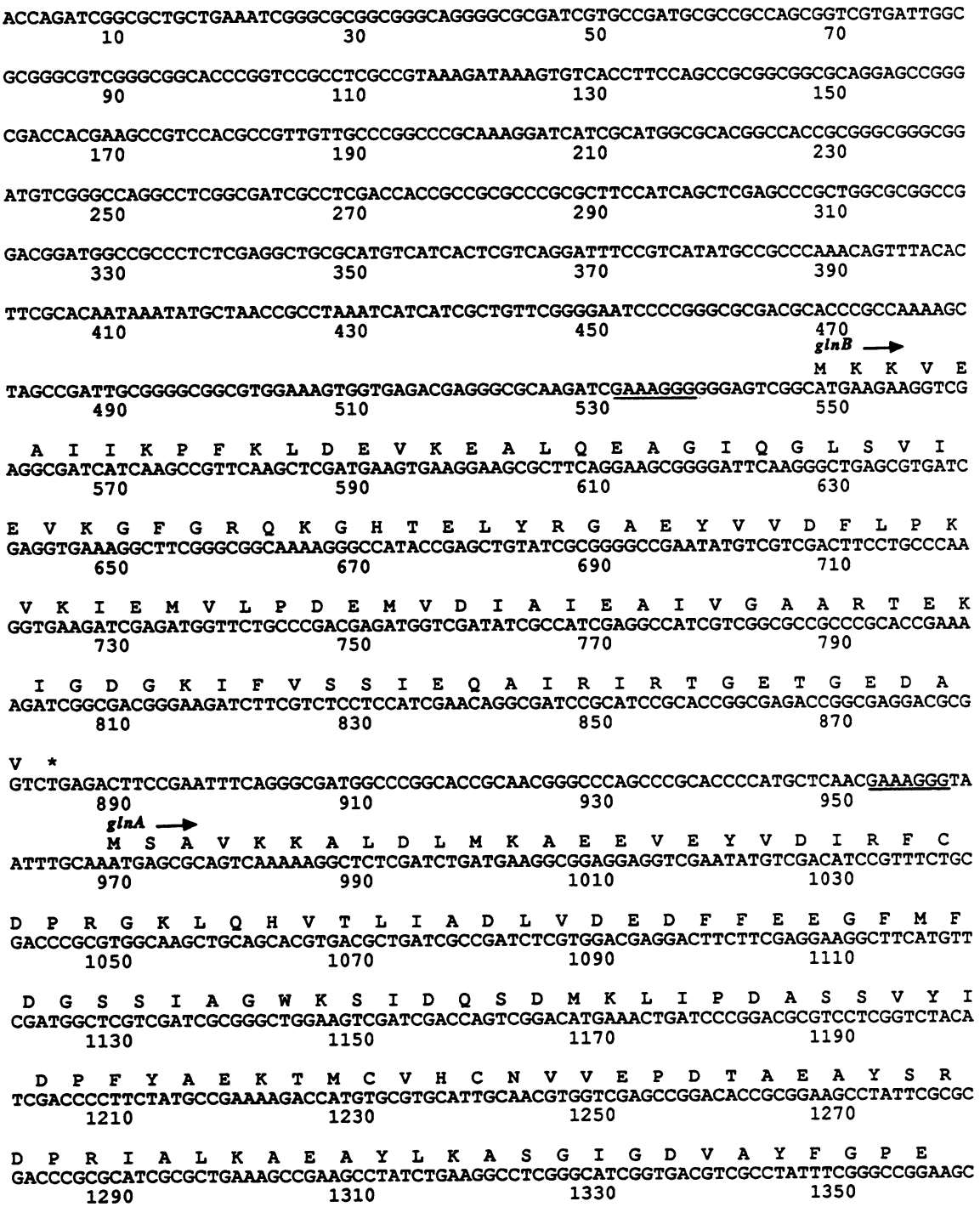

FIG. 2. Nucleotide sequence of the *glnB* gene. The DNA sequence is presented in the 5′ to 3′ direction. A total of 1,360 bp are shown. The predicted amino acid sequence of *Rhodobacter capsulatus glnB* and part of *glnA* are shown by single-letter code. A translational stop codon of *glnB* is marked with an asterisk. Designations of *glnB* and *glnA* are described in the text. Putative ribosome-binding sites are underlined. These data have been submitted to GenBank and have been assigned the accession number M28244.

vate the repressor function of *glnB* were used. If the *Rhodobacter capsulatus glnB* gene is in an operon with *glnA*, then a *glnB*::Tn*5* recombined into the chromosome would potentially result in polarity on *glnA* and glutamine auxotrophy. By using glutamine as a source of fixed nitrogen and a *glnB*::Tn*5* insertion in pRCN300 (designated pRCN548), recombination into the chromosomal copy of *glnB* could be selected. Some of the resultant Kan^r Gen^r exconjugates were glutamine auxotrophs (see below).

To verify that *glnB*::Tn*5* recombined into the chromosome, Southern hybridization was carried out by using the 2.2-kb *Eco*RI fragment containing *glnB* as a probe (Fig. 4).

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                                                                  UMP
                                                                   |
CONS   MKK  AIIK PFKLD V        G  G   V E KGFGRQ KGHTELYRGA EY VDFLPKV
R.c.   MKKVEAIIK PFKLDEVKEA LQEAGIQGLS VIEVKGFGRQ KGHTELYRGA EYVVDFLPKV
K.p.   MKKIDAIIK PFKLDDVREA LAEVGITGMT VTEVKGFGRQ KGHTELYRGA EYMVDFLPKV
E.c.   MKKIDAIIK PFKLDDVRER LAEVGITGMT VTEVKGFGRQ KGHTELYRGA EYMVDFLPKV
R.l.   MKKIEAIIK PFKLDEVRSP SG-VGLQGIT VTEAKGFGRQ KGHTELYRGA EYVVDFLPKV
B.j.                                                          ...VDFLPKV

CONS   K E V  D          I      T  IGDG KIFV       RIRTGE     A
R.c.   KIEMVLPDEM VDIAIEAIVG AARTEKIGDG KIFVSSIEQAIRIRTGETGE DAV
K.p.   KIEIVVTDDI VDTCVDTIIR TAQTGKIGDG KIFVFDVARVIRIRTGEEDD AAI
E.c.   KIEIVVPDDI VDTCVDTIIR TE-TGKIGDG KIFVFDVARVIRIRTGEEDD AAI
R.l.   KVEVVLADEN AEAVIEAIRK AAQTGRIGDG KIFVSNVEEVIRIRTGETGI DAI
B.j.   KIEIVIGDDL VERAIDAIRR AAQTGRIGDG KIFVSNIEEAIRIRTGESGL DAI
A.b.                            ....DG KIFVTPVEEVVRIRTGEKGG EAI
Er.c.      ....DI VDTCVETIMS TAQTGKIGDG KIFVFDVARVIRIRTGEEDE AAI
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FIG. 3. Comparison of the *Rhodobacter capsulatus* (R.c.) *glnB* amino acid sequence with homologous gene products from *K. pneumoniae* (K.p.), *Escherichia coli* (E.c.), *Rhizobium leguminosarum* (R.l.), *B. japonicum* (B.j.), *Azospirillum brasilense* (A.b.), and *Erwinia chrysanthemi* (Er.c.). Sequences are aligned for optimal matches, and completely conserved amino acids are indicated (CONS). UMP designates the site of uridylylation, tyrosine-51. Sequences in all cases are from translated DNA sequences as described in the text. The *Escherichia coli* sequence is corrected, as noted previously (28).

Chromosomal DNAs from two Kan^r Gen^r glutamine auxotrophs (Fig. 4, lanes 2 and 3), one Kan^r Gen^r glutamine prototroph (Fig. 4, lane 1), and SB1003 (Fig. 4, lane 4) were cut with *Eco*RI and probed. Since the 5.7-kb Tn*5* fragment does not possess *Eco*RI sites (3), the *glnB Eco*RI fragment containing Tn*5* migrated at 7.9 kb. Only the two glutamine auxotrophs showed a single 7.9-kb fragment, indicating recombination of the Tn*5* fragment into the chromosomal *glnB* gene and loss of the functional *glnB* gene. Wild-type SB1003 showed the original 2.2-kb *Eco*RI fragment. A Kan^r Gen^r glutamine prototroph showed two hybridizing fragments, one at 7.9 kb and one at 2.2 kb, suggesting that the original plasmid integrated elsewhere. This isolate was not characterized further.

Constitutive production of *nif* gene products in *glnB* insertion mutants. *Rhodobacter capsulatus glnB*::Tn*5* insertion isolate KR548, mutant 51nif^c, and wild-type SB1003 were grown in RCV-glutamine under anaerobic and aerobic conditions. Polypeptides in extracts from each of these cultures were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with both GS (Fig. 5A) and nitrogenase reductase Fe protein (Fig. 5B) antisera. When 10 μg of total protein from SB1003 was blotted with anti-GS (Fig. 5A, lane 1), a major band was observed at approximately 60,000 daltons. A minor band appeared slightly above GS in all other lanes containing 100 μg of protein. We do not know whether this minor band was due to a cross-reacting GS-related polypeptide or to a contaminating antibody in the antiserum that was used. Extracts of SB1003 and 51nif^c showed similar amounts of the GS polypeptide in cells grown aerobically (Fig. 5A, lanes 2 and 4) or anaerobically (Fig. 5A, lanes 3 and 5). The *glnB*::Tn*5* glutamine auxotroph KR548 showed less than 10% the level of GS seen in SB1003 (Fig. 5A, lanes 6 and 7).

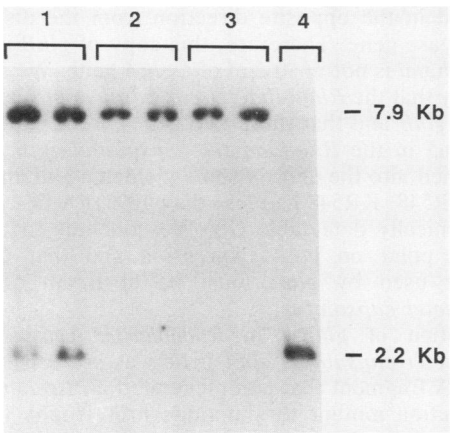

FIG. 4. Southern hybridization analysis of chromosomal DNA from *Rhodobacter capsulatus* strains by using a *Rhodobacter capsulatus glnBA* probe. DNA was digested with *Eco*RI and probed with the 2.2-kb *Eco*RI fragment (shown in Fig. 1). DNA in each lane (some duplicated) was from the following strains: Kan^r, Gen^r glutamine prototroph (lane 1), separate isolates of KR548, Kan^r, Gen^r *glnB*::Tn*5* glutamine auxotrophs (lanes 2 and 3), and strain SB1003 (lane 4).

When the same extracts were blotted with nitrogenase reductase Fe protein antisera, SB1003 and 51nif^c showed no immunoreactive polypeptides at 33,000 daltons (Fig. 5B, lanes 1, 2, 4, and 5). 51nif^c contains a *nifH-lacZYA* fusion which interrupts the chromosomal *nifHDK* operon (41). Thus, no Fe protein was made in 51nif^c even under derepressing conditions. Only KR548, when grown under anaerobic conditions, synthesized Fe protein in the presence of glutamine (Fig. 5B, lane 3). Aerobically grown KR548 showed no immunoreactive Fe protein (Fig. 5B, lane 6). Identical results were obtained when the extracts were immunoblotted with antiserum to the nitrogenase MoFe protein (data not shown).

Transcriptional analysis of *Rhodobacter capsulatus glnBA* by using a *lacZ* gene fusion. We constructed a *Rhodobacter capsulatus glnBA-lacZ* fusion plasmid, pRKR5GS, to study regulation of this operon in response to fixed nitrogen and specific regulatory proteins. pRKR5GS was conjugated into SB1003, a *nifR1* mutant (J61), and a *nifR4* mutant (LJ1). Cells containing this plasmid were induced anaerobically with and without fixed nitrogen, and extracts were assayed for β-galactosidase activities (Table 2). In the wild-type strain SB1003, β-galactosidase activity from pRKR5GS in-

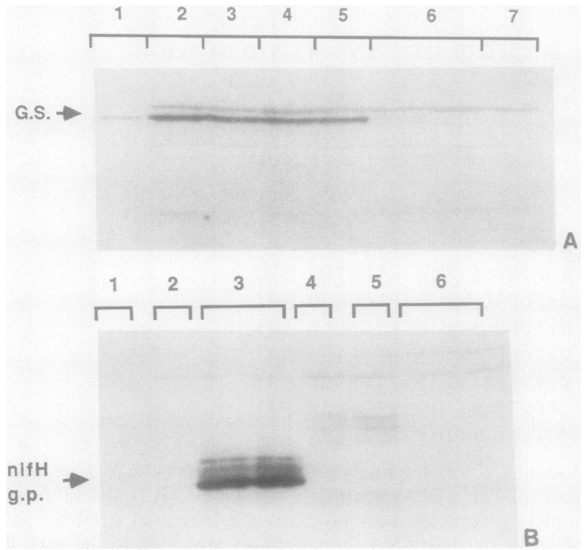

FIG. 5. Western immunoblot with GS antiserum (A) and nitrogenase Fe protein antiserum (B) against cell extracts from selected strains induced under different growth conditions. (A) Protein (100 μg) was loaded in each lane (except lane 1, in which 10 μg was loaded). Strains and growth conditions were SB1003 and anaerobic (lane 1) SB1003 and aerobic (lane 2), SB1003 and anaerobic (lane 3), 51nif^c and aerobic (lane 4), 51nif^c and anaerobic (lane 5), KR548 and aerobic (lane 6), and KR548 and anaerobic (lane 7). In lanes 2 through 5, two bands were observed at approximately 60,000 daltons. The major immunoreactive polypeptide was the lower band (lane 1) and is labeled G.S. It is unknown what the upper band represents (see text). (B) Strains and growth conditions were SB1003 and anaerobic (lane 1), 51nif^c and anaerobic (lane 2), KR548 and anaerobic (lane 3), SB1003 and aerobic (lane 4), 51nif^c and aerobic (lane 5), and KR548 and aerobic (lane 6). The nitrogenase Fe protein that migrated at 33,000 daltons is labeled nifH g.p.

creased approximately twofold when it was induced under nitrogen-limiting compared with that when it was induced under nitrogen-sufficient conditions. LJ1(pRKR5GS) showed a similar increase, whereas J61(pRKR5GS) showed no increase when it was induced under identical nitrogen-limiting conditions. The β-galactosidase activities of SB1003 (pRKR5GS) from aerobically grown cells were similar to those of cells grown anaerobically in nitrogen-sufficient media (data not shown).

TABLE 2. β-Galactosidase expression in *Rhodobacter capsulatus* strains with pRKR5GS[a]

Strain	Relevant genotype	Induction condition[b]	β-Galactosidase activity[c]
SB1003	Wild type	$+NH_3$	2,900
SB1003	Wild type	$-NH_3$	5,900
J61	*nifR1*	$+NH_3$	2,400
J61	*nifR1*	$-NH_3$	1,800
LJ1	*nifR4*	$+NH_3$	2,300
LJ1	*nifR4*	$-NH_3$	4,500

[a] pRKR5GS contained a *glnBA-lacZ* fusion.
[b] Bacteria were grown in *Rhodobacter capsulatus* basal medium and induced overnight anaerobically under the indicated condition as described previously (40).
[c] β-Galactosidase activities are in nanomoles of *o*-nitrophenol formed per minute per milligram of protein and are averages of four different inductions, with each induction showing exactly the same pattern of expression when one strain was compared with another and induced with and without ammonia.

DISCUSSION

Primary structure of *Rhodobacter capsulatus glnB*. The *nifR5* locus was cloned by conjugating a cosmid bank containing wild-type *Rhodobacter capsulatus* DNA into Nif$^{c:NH_4}$ mutants and then screening for a cosmid that was able to restore normal *nif* gene regulation to these mutants (i.e., the ability to repress *nif* transcription in the presence of fixed nitrogen). *Rhodobacter capsulatus* 51nif^c, 53nif^c, and 54nif^c were able to repress anaerobically the synthesis of nitrogen fixation genes only when they possessed pRCN300 (42). We showed here that the *nifR5* ORF is highly homologous to *glnB* from *Escherichia coli*, *K. pneumoniae*, and *Rhizobium leguminosarum*. To our knowledge, the specific effects of *glnB* mutations on *nif* gene expression in *K. pneumoniae* or other diazotrophs have not been reported. In *Escherichia coli* and *Klebsiella aerogenes*, null mutants in *glnB* show constitutive expression of the unlinked *glnA* gene (6, 21). This *ntr*-controlled *glnA* gene is normally repressed at least sevenfold in the presence of glutamine (52). Thus, *Rhodobacter capsulatus* GLNB is both physically and, with respect to the constitutive phenotype of Ntr-controlled transcription, functionally comparable to *Escherichia coli* GLNB. All GLNB proteins that were sequenced, including *R. capsulatus*, showed the conserved tyrosine-51 residue, suggesting that the mechanism of information transfer relating fixed nitrogen status to different effector proteins may be conserved. Either uridylylation of GLNB at tyrosine-51 under nitrogen-limiting conditions or genetic inactivation of GLNB resulted in the expression of *ntr*-controlled genes (see below).

Operon structure of *glnBA* in *Rhodobacter capsulatus*. In *Escherichia coli* and *K. pneumoniae*, the *ntr*-regulated gene *glnA* is in a *glnA-ntrBC* operon (44, 52). In *Rhizobium leguminosarum*, *B. japonicum*, and *Azospirillum brasilense*, *glnA* is distal to an ORF that is homologous to *glnB* (5, 9, 12). In the plant pathogen *Erwinia chrysanthemi*, the DNA sequence containing the gene for pectin methyl lyase (54) was also determined to possess the 3′ region of an ORF that would code for a GLNB-like protein. This gene would be translated in the opposite direction from the distal pectin methyl lyase gene. Therefore, this copy of *glnB* in *Erwinia chrysanthemi* is not upstream of a *glnA* gene. We showed in this study that the *Rhodobacter capsulatus glnA* gene is also distal to *glnB* and that these two genes are separated by 83 bp. A Tn*5* in the *Rhodobacter capsulatus glnB* gene that recombined into the chromosome yielded a glutamine auxotroph, KR548. KR548 had less than 10% of wild-type levels of antigenically detectable GS. We conclude that this Tn*5* insert is polar on *glnA* expression and that the major promoter used by *glnA* must be upstream of *glnB* in *Rhodobacter capsulatus*.

Regulation of *glnBA* in *Rhodobacter capsulatus*. The *Rhodobacter capsulatus glnA* gene was previously isolated as a DNA fragment that complemented an *Escherichia coli glnA* deletion mutant to glutamine prototrophy (59). That isolation required selection for mutations that presumably allowed better expression of *Rhodobacter capsulatus glnA* in *Escherichia coli*. Interpretation of regulatory studies with that DNA fragment would therefore be complicated. A study of eight glutamine auxotrophs of *Rhodobacter capsulatus* indicated that all the mutations were closely linked (64) and that each mutant synthesized *nif* proteins. Similar analyses of *Escherichia coli* and *S. typhimurium* glutamine auxo-

trophs indicated that some are mutated in *ntr* genes but not in *glnA* (43, 52). Based on these results it is not clear whether an *ntr*-like regulatory system (i.e., positive activator gene products required for optimal expression of GS) exists in *Rhodobacter capsulatus*. The *Rhizobium leguminosarum glnBA* region possesses an *ntrA* consensus-binding site upstream of *glnB* (12). Thus, it would be predicted that, like the *Escherichia coli* and *K. pneumoniae glnA-ntrBC* operons, at least some of the expression from this promoter is under *ntr* control. The DNA sequence of the *Rhodobacter capsulatus glnBA* operon reported here does not include a consensus *ntrA*-binding site. In addition, there was only a twofold increase in β-galactosidase expression from the *Rhodobacter capsulatus glnBA-lacZ* fusion when SB1003(pRKR5GS) was induced under nitrogen-limited compared with that when it was induced under nitrogen-sufficient conditions. This result agrees with the slight difference in GS levels detected by rocket immunoelectrophoresis of *Rhodobacter capsulatus* extracts (57). The twofold increase in *glnBA*-directed β-galactosidase activity under nitrogen-limiting conditions was not observed in the *nifR1* mutant J61 but was observed in the *nifR4* mutant LJ1. This result suggests that NIFR1, which is homologous to *Escherichia coli* NTRC, may be responsible for some (50%) of the *glnBA* transcription in *Rhodobacter capsulatus*. Still unexplained is the essentially wild-type level of GS activity reported for J61 incubated under nitrogen-limiting conditions (56). Strain J61 (64) and *nifR1* insertion mutants (40) are able to grow with ammonia and other sources of fixed nitrogen. It is clear that the major phenotypic effect of the *nifR1* mutations is the inability to transcribe *nif* genes (40) (see below) and possibly a methylammonium transporter gene (56). Possibly, other NTRC-like proteins are required for expression of other nitrogen-sensitive operons in *Rhodobacter capsulatus* (42). It is also possible that some genes commonly referred to as *ntr* or nitrogen-regulated genes in enteric organisms are only partially nitrogen sensitive in photosynthetic bacteria. The results reported here suggest that *Rhodobacter capsulatus glnA* may fit into this class.

***Rhodobacter capsulatus glnB* and the *Rhodobacter capsulatus* nitrogen fixation regulatory circuit.** In *Escherichia coli*, GLNB interacts with two different proteins in independent reactions. When GLNB is deuridylylated (via uridylyltransferase when the glutamine/α-ketoglutarate ratio is high), it interacts with adenylyltransferase, which subsequently adenylates and inactivates GS (for a review, see reference 60). Deuridylylated GLNB also interacts with NTRB, which then dephosphorylates and inactivates NTRC. GLNB is nonfunctional when it is uridylylated (via uridylyltransferase when the glutamine/α-ketoglutarate ratio is low). Under this condition adenyltransferase deadenylates GS, activating it, and NTRB phosphorylates NTRC. Since *Rhodobacter capsulatus* (32) and other photosynthetic bacteria (50) have been shown to possess a GS adenylylation-deadenylylation system, it is reasonable to suggest that the *glnA*-linked *glnB* gene characterized in this study is involved in that regulatory system as well.

Interaction of GLNB with NTRB must also occur in *Rhodobacter capsulatus*. Two *Rhodobacter capsulatus* regulatory genes, called *nifR1* and *nifR2*, are homologous to *Escherichia coli ntrC* and *ntrB*, respectively (34, 40). Based on the results presented here and on comparisons with the *Escherichia coli* system, it is likely that *Rhodobacter capsulatus* GLNB, in the deuridylylated state, interacts with NIFR2, with the subsequent desphosphorylation of NIFR1 (Fig. 6). Thus, only when fixed nitrogen is limiting is NIFR1

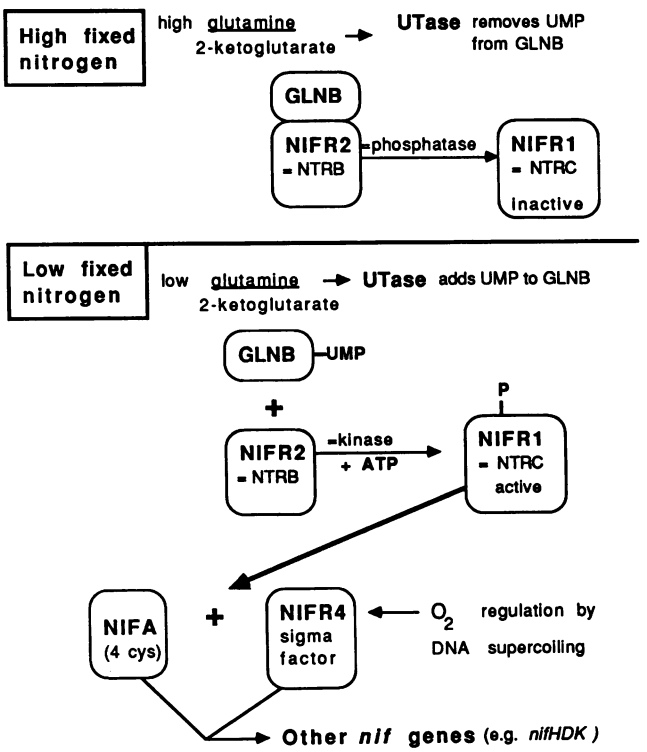

FIG. 6. *Rhodobacter capsulatus* nitrogen fixation regulatory model. The model is based in part on the work presented here and is described in the text. UTase is uridylyltransferase.

phosphorylated by NIFR2. The latter occurs only when *Rhodobacter capsulatus* GLNB is uridylylated or the *glnB* gene is inactivated. In previous studies we showed that ethyl methanesulfonate-derived *Rhodobacter capsulatus* mutants that map in *glnB* show constitutive production of *nif* products, irrespective of the exogenous glutamine concentration (41). These mutants, 51nif^c, 53nif^c, and 54nif^c, are not glutamine auxotrophs and produce normal amounts of GS (Fig. 5A). Even in the presence of exogenous glutamine, the *Rhodobacter capsulatus glnB*::Tn*5* insertion mutant KR548 synthesized *nif* proteins anaerobically (Fig. 5B). Previously described *Rhodobacter capsulatus* glutamine auxotrophs repressed the synthesis of *nif* proteins and *nif* mRNA when they were supplied with exogenous glutamine (59, 64). Therefore, the *nif* constitutive phenotype of KR548 (and of strains 51nif^c, 52nif^c, and 54nif^c) is probably due to the direct inactivation of *glnB*, presumably because NIFR2 and GLNB do not interact and NIFR1 remains active. Although no uridylyltransferase has yet been genetically or biochemically characterized, this model predicts its presence in *Rhodobacter capsulatus*. We have shown here that the proposed site of uridylylation, tyrosine-51, is conserved in *Rhodobacter capsulatus* GLNB.

Aside from some transcription from the *glnBA* operon, NIFR1 has been shown to be essential only for nitrogen fixation gene transcription (40) and methylammonium uptake (56) in *Rhodobacter capsulatus* grown under nitrogen-limiting conditions. We have recently observed that the *Rhodobacter capsulatus nifA* gene (copy II) strictly requires NIFR1 but not NIFR4 for transcription (R. G. Kranz and V. M. Pace, unpublished data). We do not yet know what the DNA recognition site is for NIFR1, nor do we know what RNA polymerase sigma factor is involved in these

interactions. A similar situation may exist in *Rhizobium meliloti*, for which the *nifA* gene is activated by another NTRC-like protein coded by *fixJ* (13, 24). The RNA polymerase holoenzyme it recognizes is not yet known (13, 24). Since NIFR1 is required for transcription of both *Rhodobacter capsulatus nifA* (copy II; 100%) and *nifR4* (at least 75% [Kranz and Pace, unpublished data]), a second level of control occurs at this point. Expression of *nif* genes in *Rhodobacter capsulatus* is inhibited by both oxygen and DNA gyrase inhibitors (41). This oxygen control occurs even when the first level of sensing and repression is missing via *glnB* or other *nif*(Con) mutations (41; this study, Fig. 5). It was postulated that the anaerobic requirement for DNA supercoiling occurs at this level (41). In addition, a second point of oxygen control may occur at the level of the *nifA* protein (46). Four conserved cysteine residues that potentially bind an oxygen-sensitive metal may modulate the activation function of NIFA (20, 29, 46). Consensus NIFA- and NTRA (i.e., NIFR4)-binding sites are present in the upstream regions of a number of *Rhodobacter capsulatus nif* genes (33, 46; R. G. Kranz, unpublished data).

The *Rhodobacter capsulatus nif* regulatory model depicted in Fig. 6 involves two levels of control. The first level senses and relays the nitrogen status in the cell, via GLNB, to repression or activation of the second-level genes (i.e., *nifR4* and *nifA*). It is postulated that the second level of control involves the response to oxygen within the cell, which is possibly modulated by DNA supercoiling and direct protein inactivation.

Similarities and differences of the *Rhodobacter capsulatus nif* regulatory cascade to other microorganisms. It was shown here that the fixed nitrogen-sensing circuit in *Rhodobacter capsulatus* is similar to the *Escherichia coli ntr* system (and, presumably, that of *K. pneumoniae*). At least three points of control in the *Rhodobacter capsulatus nif* regulatory cascade appear to differ from the *K. pneumoniae* system. First, NIFR1 (NTRC-like) probably interacts with an RNA polymerase sigma factor that is different from the NTRA-like (i.e., NIFR4) sigma factor. Second, as in some symbiotic diazotrophs, NIFA may be oxygen sensitive. Third, a nitrogen fixation-specific RNA polymerase sigma factor, encoded by *nifR4* and linked to *nifHDK*, is required in *Rhodobacter capsulatus* (2, 34, 40). The latter situation is unique to *Rhodobacter capsulatus*.

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